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Toenail selenium and risk of hepatocellular carcinoma mortality in Haimen City, China

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Selenium (Se) is an essential trace mineral with known anticarcinogenic properties in humans. However, few studies have examined the association between Se nutrient status and risk of liver cancer. We conducted a nested case-control study comparing the Se content in toenail clippings of 166 individuals (154 men, 12 women) with hepatocellular carcinoma (HCC) to 394 healthy controls (360 men, 34 women) in Haimen City, China, where HCC is a leading cause of mortality. Toenail Se concentration was measured by inductively coupled plasma-optical emission spectroscopy. Median toenail Se was lower for HCC cases than controls ($p = 0.03$). Adjusted odds ratios and 95% confidence intervals for HCC mortality by increasing quartile of toenail Se were 1.00 (reference), 0.58 (0.32–1.03), 0.83 (0.48–1.42) and 0.50 (0.28–0.90), with a marginally significant trend in risk observed (p for trend = 0.06). This inverse association appeared stronger among those who did not consume alcohol and among women. Future studies are needed to examine the interrelationship between Se, viral hepatitis infection and HCC in order to better understand the etiologic mechanisms involved and evaluate the true chemopreventive potential of Se compounds for liver diseases.
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With poor prognosis for survival, hepatocellular carcinoma (HCC) ranks as the third leading cause of global cancer mortality, resulting in approximately 529,000 deaths each year.[1] In China, where 54% of all HCC cases are diagnosed,[1] there is great interest in the chemopreventive utility of selenium (Se) for reducing the burden of cancer, given evidence that Se is an essential trace mineral with anticarcinogenic properties. It is well established that Se is an integral component of functionally active selenoproteins, including the glutathione peroxidases (GPX), known to protect cells against genetic damage by free radicals. The importance of selenoproteins in proper liver function has been supported by recent experimental data showing that hepatocellular degeneration and necrosis result when selenoprotein synthesis is blocked in murine hepatocytes.[2] Lower levels of both liver Se-dependent GPX activity and serum Se concentration have also been noted in HCC patients relative to healthy adults,[3] although it is uncertain whether these changes influence or reflect disease progression.

To date, few studies have examined the association between Se nutrient status and risk of liver cancer in humans. Ecologic studies in Qidong City, China, have revealed inverse relationships between incidence of primary liver cancer and both the Se content of grains and Se levels of pooled blood samples.[4] In contrast, ecologic surveys across 65 Chinese counties have not demonstrated a strong correlation between plasma Se levels and liver cancer mortality.[5,6] Several interventional trials also conducted in Qidong City have suggested that Se supplementation is effective in reducing rates of primary liver cancer in populations at high risk.[7–9] Similarly, a nested case-control study of men with chronic hepatitis B infection in Taiwan showed an inverse, yet nonlinear association between plasma Se and HCC risk, particularly among cigarette smokers and those with low plasma retinol or carotenoid levels.[10]

Although such research has yielded fairly consistent results, it has been primarily conducted in Qidong City, suggesting the need for confirmation in other populations. In 1992, a large prospective cohort study was launched in Haimen City, China, to assess specific environmental, viral and genetic risks associated with HCC. Haimen City is located adjacent to Qidong City in the Jiangsu Province of China, a region where HCC incidence and mortality are among the highest in the world. We designed a nested case-control study within the Haimen City cohort to investigate whether Se nutrient status, as determined by toenail Se levels, is related to HCC risk in both men and women. For estimating the influence of Se on subsequent disease occurrence, toenail analysis offers a better method to assess individual Se status than measurement either in blood or by dietary questionnaire, since toenail Se concentration reflects long-term average Se intake for a period of 26–52 weeks.[11]

Material and methods

Cohort enrollment and follow-up

Study protocol and materials were approved by the Institutional Review Board of Fox Chase Cancer Center, the Medical Ethics Review Group of Haimen City and the Ethics Review Committee of Shanghai Medical University. Methods for enrollment of the baseline cohort have been previously described.[12] Briefly, from January 1992 to December 1993, trained personnel from the Haimen City Anti-Epidemic Station (HCAS; now called the Haimen Center for Disease Control) visited 1,008 villages across the 35 townships of Haimen City to recruit and enroll study participants. Each participant, upon providing written consent, was asked to engage in a brief interview and donate a 9.0 ml sample of blood by venipuncture and 5 drops of blood on filter cards. The interview included completing a one-page structured questionnaire to elicit information on sex, date of birth, current residence, height, weight, occupation, cigarette use, alcohol consumption, tea drinking, past pesticide exposure, history of drinking water source, history of staple food (corn, rice and wheat) consumption, selected medical conditions and family history of HCC. Starting in January 1993, the study protocol also included the collection of toenail clippings by study interviewers using ceramic nail clippers. There were no

Abbreviations: GPX, glutathione peroxidase; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCAS, Haimen City Anti-Epidemic Station; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; ICP-OES, inductively coupled plasma-optical emissions spectroscopy; NAA, neutron activation analysis; ppm, parts per million; RIA, radioimmunoassay; Se, selenium.

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refusals among the participants who were asked to provide toenail clippings ($n = 41{,}563$). In all, 90,836 adult residents of Haimen City comprised the entire cohort at baseline.

Since study entry, follow-up on vital status and HCC incidence of cohort members has been ongoing. Township and citywide referral hospitals are required each month to submit reports of all cancer diagnoses and deaths from any cause for residents within their jurisdiction to the HCAS. These reports are evaluated annually by Haimen City public health authorities to ensure their completeness and accuracy. In addition, study personnel continue to verify death certificate information for cohort members on a biannual basis by contacting township and village physicians and by reviewing existing medical records. All diagnoses of HCC are confirmed by medical record review and, for those who have died, through contact with their physicians and family members. As of 31 December 2002, deaths have been primarily reported on the basis of death certificates completed by physicians, with 68.8% from citywide referral hospitals and 26.1% from local township hospitals; the remainder has been ascertained through contact with village physicians and family members.[13]

Study population

Participant selection for this nested case-control study occurred from follow-up until 30 September 2000. All cohort members diagnosed with HCC who provided questionnaire data, a blood sample and toenail clippings were included in the case group for a total of 445 cases (408 men and 37 women). Diagnosis of HCC was confirmed by elevation of serum α-fetoprotein level ($>$ 400 ng/ml) in 1.5%, imaging (ultrasonography, computerized tomography, or magnetic resonance imaging) in 39.5%, both α-fetoprotein elevation and imaging in 35.8%, clinical criteria in 1.7% and death certificate and/or *postmortem* interview with physicians or family in 21.5% of the cases. In addition to these criteria, 8.8% of the cases were confirmed by histologic examination.

From the study cohort, controls were randomly selected and frequency-matched on age, sex and township of residence to HCC cases. Controls who tested positive for hepatitis B surface antigen (HBsAg) were oversampled in order to ensure ability to examine the relationship between selenium and HCC independent of HBsAg status. All 1,383 selected controls completed the study questionnaire and provided a blood sample and toenail clippings at enrollment.

HBsAg testing

Within 24 hr of blood collection, serum was separated from whole blood for HBsAg testing by radioimmunoassay (RIA) at the HCAS. Serum samples from a subset of cohort members were subsequently shipped to Fox Chase Cancer Center and retested for HBsAg with a commercial enzyme immunoassay kit (AUSZYME; Abbott Laboratories, North Chicago, IL). As recently reported by Evans *et al.*,[14] the sensitivity and specificity of the RIA was 88.9% and 95.2%, respectively, when judged against AUSZYME for 9,360 serum samples. Given that HBsAg testing was conducted at Fox Chase Cancer Center for only a subset of the cohort, HBsAg status was defined exclusively on HCAS test results.

Toenail analysis

Toenail analyses were conducted at the Nutrient and Elemental Analysis Laboratory at Cornell University (Ithaca, NY). Samples were analyzed in multiple batches over the follow-up period as participants were identified for study inclusion. In total, 16 batches were run: 13 between March 1995 and September 1997 and 3 between September and December 2000. Laboratory personnel were blinded to case-control status.

Individual samples of toenail clippings were first decomposed by microwave digestion.[15] For the earlier batches, toenail samples were individually weighed into 50 ml Pyrex tubes and digested with 5 ml of a 2:1 mixture of ultrapure nitric acid and perchloric acid. This step was later modified for batches run in 2000, in that samples were weighed and sealed into Teflon vessels with 10 ml of ultrapure nitric acid. The Mars 5 microwave system (CEM, Matthews, NC) was consistently operated under power and pressure control parameters of 1,200 watts and 150 psi, respectively. Its unit temperature was ramped over 20 min to 210°C, at which it was maintained for another 10 min; specifically for the nitric acid and perchloric mixture, digestion was conducted at 210°C until white fumes were emitted. In each cycle, 13 vessels were processed simultaneously, and a blank sample was included as an internal control. This preparation method resulted in a complete digestion of the clippings without need for filtration. Digests were then diluted with distilled deionized water to a 45 ml volume.

Inductively coupled plasma-optical emission spectroscopy (ICP-OES) was performed on each digested sample to analyze its elemental content with a Thermo Elemental IRIS ICP-OES duoview spectrophotometer equipped with a concentric nebulizer and cyclonic spray chamber. The samples were run at an 1,150 kilowatt power setting using a Y-internal standard and background correction. Three measurements of each element were acquired per sample, and the mean toenail Se concentration (unit: parts per million, ppm) was derived by averaging the Se values obtained for each individual. Se recovery values through the digestion process were examined after spiking samples with Se. A mean recovery rate of 93% was obtained.

Statistical analysis

A modified preparation method of toenail samples for elemental content analysis was implemented in 2000, resulting in a substantial difference in the range of Se concentration values obtained between earlier and later batches. Specifically, the interquartile range of toenail Se was wider for samples analyzed prior to 2000, an indication of larger measurement variability using the first nail digestion method. We therefore calculated and compared the coefficient of variation (CV) for Se from toenail samples analyzed before and during 2000. Since only the CV for samples analyzed in 2000 (30.6%) was acceptable, we excluded individuals with toenail Se values obtained before 2000, leaving 166 HCC cases (154 men, 12 women) and 394 controls (360 men, 34 women) in our final analysis.

In Haimen City, the rates of HCC incidence and mortality are nearly equivalent as the median duration of survival is less than 6 months. Mortality was selected as the outcome of interest rather than incidence in our analysis in order to reduce potential biases from the differential reporting of HCC among individuals who received medical care outside of Haimen City. Besides evaluating the overall association between toenail Se and risk of HCC mortality, we performed similar analyses stratified by sex because of the well-described sex-related differences in HCC incidence.[16]

Covariates of interest included the observed risk factors for HCC mortality in the overall cohort after 8 years of follow-up: HBsAg status, history of acute hepatitis, family history of HCC and occupation as a peasant.[14] Any individual who primarily engaged in agricultural work was defined as a peasant. Other covariates identified *a priori* were cigarette smoking and alcohol use, factors that have been shown to influence Se concentration in blood[17,18] and toenails.[19] An individual was considered to be a smoker if he or she ever reported smoking at least one cigarette per day for longer than 6 months. Responses on smoking duration (in years) and number of cigarettes per day ($\leq$ 5, 6–10, 11–20 and $>$ 20) were also captured, from which a categorical variable for smoking in units of pack-years ($<$ 9, 10–19 and $\geq$ 20) was derived. Consumers of alcohol were those who reported drinking alcoholic beverages (beer, grain wine, grape wine, rice wine and liquor) at least 4 or more times per week. For each beverage type, the number of years and quantity per occasion consumed were recorded; these values were used to create a categorical variable of alcohol use (low, moderate and high) based on the tertile distribution of drinks consumed per person-year among controls.

Difference in the median concentration of toenail Se was examined between individuals with and without HCC using the Wilcoxon rank-sum test. Unconditional logistic regression was conducted to

generate odds ratios (ORs) and 95% confidence intervals (CIs) for estimating risk of HCC mortality associated with Se status. Individuals were categorized according to the quartile distribution of toenail Se values among controls. These same quartile cutpoints were used in conducting sex-specific analyses; however, due to the limited number of women studied, women were grouped based on whether Se values were below or above the 25th percentile mark. The aforementioned covariates were considered as potential confounding variables and entered into the final multivariate models if their inclusion resulted in at least a 10% change in the parameter estimates for toenail Se. Levels of toenail Se concentration were examined on a continuous scale to test for trend, where two-sided $p < 0.05$ was considered statistically significant. Stratified analyses were also performed to examine potential effect modification by HBsAg status, smoking history and alcohol use. All analyses were conducted with SAS version 8.2 software (SAS Institute, Cary, NC).

Results

Baseline characteristics for participants of the nested case-control study are shown in Table I. There were significant case-control differences in the distributions of HBsAg status and history of acute hepatitis for men and women, with more cases being HBsAg-positive and having a history of acute hepatitis than controls. Differences by disease status were also observed for alcohol use, occupation and family history of liver cancer among men. Across sex, a much greater percentage of men reported being regular consumers of cigarettes and alcohol than women.

Overall and sex-specific median, mean and interquartile range values of toenail Se concentration are shown in Table II. Cases had significantly lower toenail Se concentration than controls ($p = 0.03$), and by sex, this difference was evident only in women. Median toenail Se concentration for men and women without HCC, however, was similar ($p = 0.59$). The average time from toenail collection until mortality among individuals who developed HCC was 5.5 ± 1.5 years.

Table III presents risk estimates of HCC mortality associated with toenail Se concentration by quartile. The final multivariate models for men and women combined were adjusted for age, sex, HBsAg status, alcohol use, history of acute hepatitis and occupation. The final multivariate models for men were adjusted for age, HBsAg status, alcohol use, history of acute hepatitis, family history of HCC and occupation, and those for women were adjusted for age, HBsAg status and history of acute hepatitis. Only individuals categorized in the highest quartile of toenail Se were at decreased risk of HCC mortality, with a borderline significant trend in risk by Se concentration (p for trend $= 0.06$). When the analysis was restricted to men, the strength of the association was attenuated. Comparing the magnitude of the sex-specific risk estimates, the influence of Se nutrient status on risk of HCC mortality appeared somewhat stronger for women than men.

When alcohol use, smoking history and HBsAg status were examined as potential modifiers of the association between Se nutrient status and HCC mortality, a strong negative association was observed among nondrinkers but not drinkers (p for interaction $=$ 0.03; Table III). This interaction by alcohol use remained evident when the analysis was limited to men. No effect modification by either smoking history or HBsAg status was found (data not shown).

Discussion

Although hepatitis B virus and hepatitis C virus are the major risk factors for HCC, other environmental, genetic and host risk factors also appear influential in the etiology of HCC. Such risk factors include aflatoxin exposure, alcohol consumption, cigarette smoking and steroid hormones.[16] Nutritional status may likewise affect HCC risk, but research on this topic has been more limited. Various mechanisms by which Se may act to impact protection against cancer have been proposed, including defense against oxidative stress, alteration of carcinogen metabolism, control of cell proliferation, induction of apoptosis, inhibition of angiogenesis and modulation of immune function.[20–22] In support, we observed that individuals with the highest Se levels were at significantly decreased risk of HCC mortality. Confounding did not appear to account for this association.

Observational studies examining the influence of Se nutrient status on subsequent risk of cancer have commonly used Se concentration of prediagnostic biologic specimens as an approximate measure of Se intake. The enormous variation of Se content in foods, reflective of distinct geographic differences in soil composition, has precluded accurate estimation of Se intake using dietary questionnaires. In this study, we measured Se concentration of toenail clippings collected from participants at baseline. Analysis of toenails, compared to serum or plasma, was preferable in this study setting, because it offered an average estimate of long-term Se exposure rather than exposure at a single snapshot in time. In spite of this difference, our findings were fairly consistent with prior studies of Se status and liver cancer in which Se concentration was measured in blood.

While sufficient levels of Se may reduce susceptibility for the development of liver cancer in high-risk populations, there has been no clear evidence of a dose-response relationship. In fact, a linear dose-response relationship between Se status and human cancer has not been consistently shown in epidemiologic investigations.[23] We observed a marginally significant decreasing trend in HCC mortality risk across quartiles of toenail Se concentration. In a previous study conducted in Taiwan, a U-shaped pattern across quintiles of plasma Se concentration was apparent, with men classified in the middle quintiles being at the lowest risk for HCC.[10] The effect of Se dosage (via supplementation) on primary liver cancer incidence was not examined in the intervention trials conducted in Qidong City, China. Such observed inconsistency could be attributed to marked differences in selected design and population across studies. Alternatively, there may be a minimum threshold at which Se influences carcinogenesis, as we noted that only individuals with the highest Se levels were at significantly lower risk of HCC mortality.

In further contrast with the Taiwan study,[10] our study population was not restricted to individuals with chronic hepatitis virus infection, even though we oversampled controls who tested positive for HBsAg to estimate risk of HCC mortality associated with Se independent of HBsAg status. While existing data support a link between Se and chronic HBV infection, it is unclear whether Se status affects susceptibility to infection or whether infection modifies the role of Se, and how either relates to HCC development. Similar to patients with HCC, patients with other viral liver diseases have relatively lower Se levels when compared to healthy individuals.[24–26] Both rates of liver cancer incidence and HBsAg positivity were significantly lower after table salt fortification with 15 ppm sodium selenite was administered in the Qidong City intervention trials.[7,9] More recently, HCC cells, in particular those with integrated HBV DNA sequences and an aflatoxin-related p53 mutation, were shown to acquire tolerance to Se deficiency *in vitro*.[27] Scientists have even proposed, using structural bioinformatics, that hepatitis C virus (HCV) can encode an Se-dependent GPX gene, suggesting that Se status is an important factor in HCV-related disease progression.[28] Examining the association between toenail Se and HCC mortality by HBsAg status, we found no remarkable difference in risk. Whether this association is truly not modified by HBsAg status, however, should be confirmed, since this null finding may be attributed to the small number of HBsAg-negative cases.

Similarly, we assessed the presence of effect modification by cigarette smoking and alcohol use, factors that have been correlated with decreased levels of Se.[17,19,29] Among study controls, median toenail Se concentration was slightly higher for nonconsumers than consumers of cigarettes or alcohol, but these differences did not reach statistical significance. Among both nonsmokers and nondrinkers, however, toenail Se concentration was notably lower in cases than controls. Although the stronger inverse relation between Se status and HCC risk among smokers noted in the Taiwan study[10]

TABLE I – BASELINE CHARACTERISTICS OF HCC CASES AND CONTROLS

	Overall			Men			Women		
	Cases	Controls	p^1	Cases	Controls	p^1	Cases	Controls	p^1
With toenail samples, *n*	166	394		154	360		12	34	
Age (years), *n* (%)									
25–34	23 (13.9)	50 (12.7)		21 (13.6)	45 (12.5)		2 (16.7)	5 (14.7)	
35–44	55 (33.1)	126 (32.0)		51 (33.1)	117 (32.5)		4 (33.3)	9 (26.5)	
45–54	53 (31.9)	142 (36.0)		49 (31.8)	129 (35.8)		4 (33.3)	13 (38.2)	
≥ 55	35 (21.1)	76 (19.3)	0.81	33 (21.4)	69 (19.2)	0.81	2 (16.7)	7 (16.7)	0.97
HBsAg status, *n* (%)									
Negative	63 (38.0)	262 (66.5)		61 (39.6)	242 (67.2)		2 (16.7)	20 (58.8)	
Positive	103 (62.0)	132 (33.5)	< 0.01	93 (60.4)	118 (32.8)	< 0.01	10 (83.3)	14 (41.2)	0.02
Smoking, *n* (%)									
Never	66 (39.8)	154 (39.1)		56 (36.4)	122 (33.9)		10 (83.3)	32 (94.1)	
Ever	100 (60.2)	240 (60.9)	0.92	98 (63.6)	238 (66.1)	0.61	2 (16.7)	2 (5.9)	0.28
< 10 pack-years	31 (18.8)	107 (27.2)		30 (19.6)	105 (29.2)		1 (8.3)	2 (5.9)	
10–19 pack-years	36 (21.8)	82 (20.8)		35 (22.9)	82 (22.8)		1 (8.3)	0 (0.0)	
≥ 20 pack-years	32 (19.4)	51 (12.9)	0.08	32 (20.9)	51 (14.2)	0.07	0 (0.0)	0 (0.0)	0.39
Alcohol consumption, *n* (%)									
No	76 (45.8)	156 (39.6)		64 (41.6)	127 (35.3)		12 (100.0)	29 (85.3)	
Yes (≥ 3 drinks/week)	90 (54.2)	238 (60.4)	0.19	90 (58.4)	233 (64.7)	0.20	0 (0.0)	5 (14.7)	0.31
Low	15 (9.0)	74 (18.8)		15 (9.7)	69 (19.2)		0 (0.0)	5 (14.7)	
Moderate	37 (22.3)	87 (22.1)		37 (24.0)	87 (24.2)		0 (0.0)	0 (0.0)	
High	38 (22.9)	77 (19.5)	0.03	38 (24.7)	77 (21.4)	0.05	0 (0.0)	0 (0.0)	0.31
Occupation,[2] *n* (%)									
Nonpeasant	32 (19.3)	215 (54.6)		32 (20.8)	207 (57.5)		0 (0.0)	8 (23.5)	
Peasant	134 (80.7)	179 (45.4)	< 0.01	122 (79.2)	153 (42.5)	< 0.01	12 (100.0)	26 (76.5)	0.09
History of acute hepatitis, *n* (%)									
No	92 (55.4)	304 (77.2)		88 (57.1)	280 (77.8)		4 (33.3)	24 (70.6)	
Yes	74 (44.6)	90 (22.8)	< 0.01	66 (42.9)	80 (22.2)	< 0.01	8 (66.7)	10 (29.4)	0.04
Family history of liver cancer, *n* (%)									
No	136 (81.9)	363 (92.1)		126 (81.8)	334 (92.8)		10 (83.3)	29 (85.3)	
Yes	30 (18.1)	31 (7.9)	< 0.01	28 (18.2)	26 (7.2)	< 0.01	2 (16.7)	5 (14.7)	0.99

[1]Calculated using chi-square test (for overall and men) or Fisher's exact test (for women).–[2]A peasant was defined as an individual who primarily engaged in agricultural work. Occupations classified as nonpeasant included factory worker, functionary (office worker), and business owner.

TABLE II – TOENAIL SELENIUM LEVELS IN HCC CASES AND CONTROLS OVERALL AND BY SEX

Toenail selenium (ppm)	Overall			Men			Women		
	Cases	Controls	p^1	Cases	Controls	p^1	Cases	Controls	p^1
Number	166	394		154	360		12	34	
Median	2.3	2.6	0.03	2.5	2.5	0.11	1.6	2.8	0.03
Mean	3.1	3.5		3.2	3.5		2.4	3.7	
Interquartile range	1.5–3.6	1.7–4.4		1.5–3.7	1.7–4.4		1.4–2.1	1.8–4.5	
Number of years since toenail collection, mean (SD)	5.5 (1.5)			5.5 (1.4)			5.7 (1.8)		

[1]Calculated using the Wilcoxon rank-sum test.

TABLE III – RISK-ESTIMATES OF HCC ASSOCIATED WITH TOENAIL SELENIUM LEVELS OVERALL AND STRATIFIED BY ALCOHOL USE FOR MEN AND WOMEN

	Overall				Nondrinkers				Drinkers			
	Cases	Controls	OR[1]	95% CI	Cases	Controls	OR[1]	95% CI	Cases	Controls	OR[1]	95% CI
Men and women ($n = 560$)												
< 1.71	54	98	1.00	Reference	29	31	1.00	Reference	25	67	1.00	Reference
1.71–2.54	36	99	0.58	0.32–1.03	18	41	0.39	0.16–0.97	18	58	0.80	0.37–1.73
2.55–4.42	47	98	0.83	0.48–1.42	20	46	0.40	0.18–0.93	27	52	1.54	0.75–3.16
≥ 4.43	29	99	0.50	0.28–0.90	9	38	0.26	0.10–0.70	20	61	0.80	0.38–1.68
		p for trend = 0.06				p for trend < 0.01				p for trend = 0.98		
Men ($n = 514$)												
< 1.71	47	90	1.00	Reference	22	25	1.00	Reference	25	65	1.00	Reference
1.71–2.54	32	92	0.56	0.30–1.03	15	34	0.30	0.11–0.83	18	58	0.77	0.36–1.66
2.55–4.42	47	88	0.89	0.51–1.58	19	38	0.35	0.14–0.89	27	50	1.45	0.71–2.95
≥ 4.43	28	90	0.57	0.31–1.05	8	30	0.28	0.10–0.83	20	60	0.84	0.40–1.74
		p for trend = 0.21				p for trend = 0.02				p for trend = 0.96		
Women ($n = 46$)												
< 1.71	7	8	1.00	Reference								
≥ 1.71	5	26	0.18	0.03–1.13								

[1]For men and women: adjusted for age, sex, HBsAg status, history of acute hepatitis, alcohol use (except in stratified analysis) and occupation. For men: adjusted for age, HBsAg status, history of acute hepatitis, alcohol use (except in stratified analysis), occupation and family history of liver cancer.–For women: adjusted for age, HBsAg status and history of acute hepatitis.

was not replicated, an unexpected interaction by alcohol use was observed, in which the benefit of Se on HCC mortality was restricted to nondrinkers. Several mechanisms have been proposed to explain why chronic drinkers have lower Se status, including insufficient intake, greater physiologic need, slower absorption and modified metabolism of Se.[30] While this finding may have been merely observed by chance, we speculate that, since drinkers are more likely to have limited liver function and possibly worse nutrition, chronic alcohol consumption might negate any positive influence that Se could have on hepatocarcinogenesis.

Another unresolved issue is whether Se levels differ by sex in humans.[17] To our knowledge, we are the first to investigate possible sex-related differences in the relationship between Se status and HCC risk. As outlined in a recent review of prospective studies on Se status and cancer risk, current evidence suggests that the influence of Se status on cancer risk is more pronounced in men than women.[31] Conversely, our data indicate that the reduction in HCC mortality risk associated with higher toenail Se levels may be greater for women, except no statistically significant difference in median toenail Se concentration was evident between male and female controls. Although this apparent distinction cannot be established with certainty given the small number of women studied, it could be reflective of innate differences in sex hormones and Se metabolism between men and women. Animal studies have demonstrated that activity of liver GPX is stimulated by estrogen[32,33] and suppressed by testosterone.[34,35] Fluctuations in plasma estradiol levels in women have also been positively correlated with erythrocyte GPX activity throughout the menstrual cycle.[36] Likewise, Se levels have been shown to vary according to estrogen status over the female lifespan,[37] with levels of estradiol and GPX activity significantly higher in premenopausal than postmenopausal women.[38] This link between estrogen and Se metabolism has been further substantiated by recent evidence of a direct correlation between estradiol, plasma Se and plasma and erythrocyte GPX activity,[39] possibly revealing why the beneficial effect of Se on HCC risk might be greater in women, especially premenopausal women. Although menopausal status was not ascertained, the majority of women studied were likely pre- or perimenopausal at baseline, given their age distribution. In addition, this sex-based difference might be linked to the striking contrast in cigarette and alcohol use between men and women in this study.

In light of these novel findings, several limitations need to be considered. Foremost, our analysis was restricted to a portion of the original study population due to substantial variability in Se measurement associated with the first toenail preparation method, thereby decreasing the statistical power by which to evaluate the association between Se status and HCC risk. In effect, cases who had died earlier in the follow-up period were preferentially excluded from the analysis, since toenail Se concentration was determined as cancer cases and matched controls were selected for study inclusion, and we were unable to evaluate whether cancer risk associated with toenail Se differed by extent of follow-up. Time measured from toenail collection to HCC mortality among cancer cases ranged from 1.0 to 9.6 (median = 5.5) years. In the Taiwan study, plasma Se was more negatively associated with HCC risk after cases who were diagnosed closer to the time of blood collection (< 2.8 years) and their matched controls were excluded,[10] indicating that bias due to the influence of preclinical disease was unlikely. Although the time interval when Se may confer the greatest benefit for preventing cancer has not been established, it has been suggested that Se exerts its chemopreventive effect in the earlier stages of carcinogenesis.[40] If true, with longer follow-up, we would expect to observe a stronger inverse relationship between Se and HCC risk. Even with the second preparation method, individual measurement variability of toenail Se concentration was evident, a bias that also potentially influenced risk estimates closer to the null.

Another limitation was imposed by measuring Se toenail concentration with ICP-OES, instead of neutron activation analysis (NAA) as done in prior studies. Since data comparing ICP-OES and NAA are currently unavailable, the mean toenail Se concentration measured in this Haimen City subcohort relative to that reported for other study populations could not be readily assessed. We did analyze a small sample of toenail clippings for other participants in the larger cohort by NAA, which confirmed that Haimen City is a region of low Se intake (data not shown). In spite of this constraint, the relative ranking of Se nutrient status among study participants was ensured by analyzing all specimens using ICP-OES. Further, data on factors that may influence the relationship between Se status and HCC risk were limited, thereby prohibiting account of all possible confounding or effect modification. For example, although no information was collected on dietary intake and use of nutritional supplements, levels of both carotenoids and retinol have been shown to affect susceptibility for HCC development inversely,[41,42] and in the Taiwan study, the inverse association between Se and HCC risk was notably stronger for men with lower plasma retinol levels.[10] Limited data also exist on how hepatocytes respond to Se deficiency and how Se influences action against HCC development. By measuring overall Se intake, we evaluated total Se content, not the function of individual Se-containing compounds, in relation to HCC risk. This may be an important distinction to examine since only certain selenoproteins may be responsible for maintaining normal liver function.

In summary, this nested case-cohort study offers evidence of an inverse association between Se nutrient status and risk of HCC mortality. The more pronounced reductions in risk observed among nondrinkers and among women merit further investigation, given the limited size of this study. While viral hepatitis is not a prerequisite for liver carcinogenesis, such infection is a primary cause of HCC, and Se appears to be related to both chronic hepatitis infection and HCC incidence. Therefore, closer examination of the interrelationship between Se, hepatitis B and C viruses and HCC is also essential for better understanding of the etiologic mechanisms involved and for evaluation of the true chemopreventive potential of Se compounds for liver diseases.

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